# The replication machinery of Clostridium difficile:a potential target for novel antimicrobials

Eijk, H.W. van

Chapter 6

# The transcriptional effects of sub–inhibitory concentrations of PolC–inhibitors suggest a gene–dosage dependent response to replication inhibition in *Clostridium difficile*


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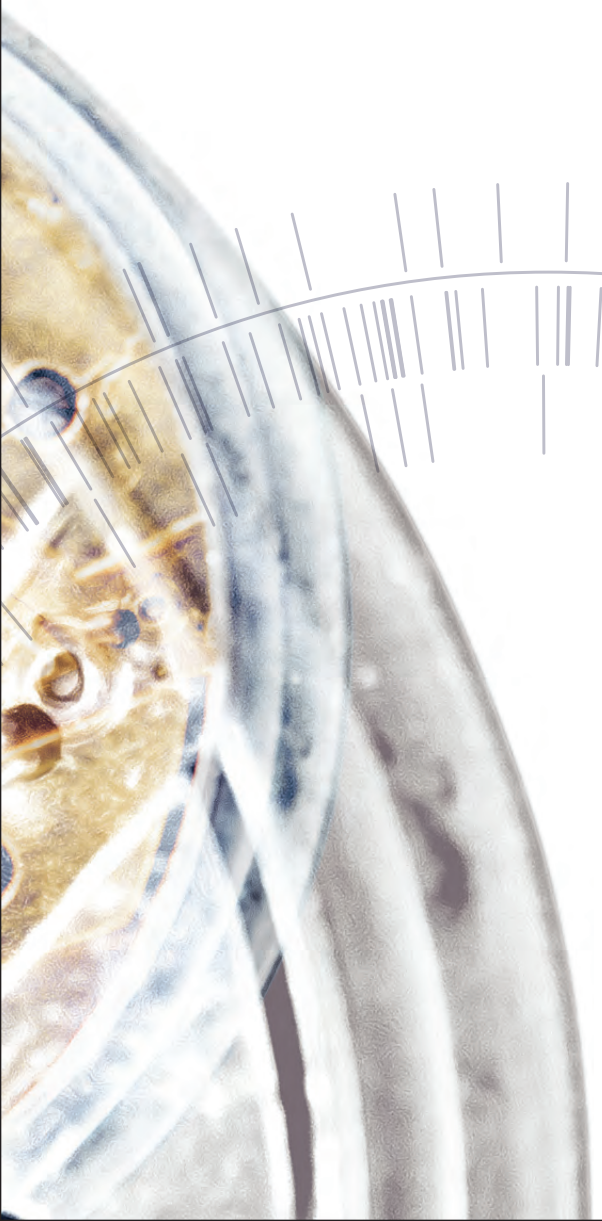


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# Abstract

***Clostridium difficile*** **is a potentially lethal gut pathogen that causes nosocomial and community-acquired infections. Limited treatment options and reports of reduced susceptibility to current treatment emphasize the necessity for novel antimicrobials. The DNA-polymerase of Gram-positive organisms is an attractive target for the development of antimicrobials. 362E (*N*$^2$-(3,4-dichlorobenzyl)-7-(2-[1-morpholinyl] ethyl) guanine; MorE-DCBG) is a DNA polymerase inhibitor in preclinical development as a novel therapeutic against *C. difficile* infection. This synthetic purine shows preferential activity against *C. difficile Pol* IIIC (PolC) over those of other organisms *in vitro* and is effective in an animal model of *C. difficile* infection. Its specificity may limit the negative effects on the colonic microbiota. In this study we have determined its efficacy against a large collection of clinical isolates. At concentrations below the minimal inhibitory concentration (MIC), the presumed slowing (or stalling) of replication forks due to 362E leads to a growth defect. We have determined the transcriptional response of *C. difficile* to replication inhibition and observed an overrepresentation of upregulated genes near the origin of replication in the presence of PolC-inhibitors, but not when cells were subjected to sub-inhibitory concentrations of other antibiotics. This phenomenon can be explained by a gene dosage shift, as we observed a concomitant increase in the ratio between origin-proximal and terminus-proximal gene copy number upon exposure to PolC-inhibitors.**

## Background

*Clostridium difficile* (*Clostridioides difficile*) [1] is a Gram-positive, anaerobic bacterium that can asymptomatically colonize the intestine of humans and other mammal [2-4]. However, when the normal flora is disturbed, *C. difficile* can overgrow and cause fatal disease, as has been dramatically demonstrated in the Stoke Mandeville Hospital outbreaks in 2004 and 2005 [5]. The ability to form highly resistant endospores coupled to its extensive antibiotic resistance have contributed to its success as a nosocomial and community-acquired pathogen [2-4]. Recent years have seen an increase in the incidence and severity of *C. difficile* infections (CDI), due to the emergence of certain PCR ribotypes [4,6]. Antibiotic use is a well-established risk factor for CDI [7], and the emergence of the epidemic PCR ribotype 027 has been linked to fluoroquinolone resistance [8]. At present, two antibiotics, metronidazole and vancomycin, are commonly used to treat CDI, and a third, fidaxomicin, is indicated for the treatment of relapsing CDI [9,10]. Clearly, limited treatment options and reports of reduced susceptibility to current treatment [11-13] emphasise the necessity for the development of novel antimicrobials and a better understanding of tolerance and resistance towards existing therapeutics.

It is increasingly realized that off-target effects, that occur when cells are exposed to antimicrobials [14], can contribute to its efficacy but also facilitate the emergence of tolerance and/or resistance. Antimicrobials may act as signalling molecules which modulate gene expression [14]. Additionally, in particular, those targeting DNA replication (such as polymerase inhibitors) can cause transcriptional effects as a result of differences in gene dosage [15].

The polymerase of Gram-positive organisms is an attractive target for the development of novel antimicrobials [16]. First, these PolC-type polymerases are absent from Gram-negative organisms and humans [17,18]. HPUra (6(*p*-Hydroxyphenylazo)-uracil), one of the first such compounds, is therefore highly active against a wide range of Gram-positive bacteria but does not affect Gram-negative bacteria [17,18]. Template-directed elongation is blocked by the inhibitor through simultaneous binding to the cytosine of the DNA strand and near the active site of PolC. Second, compounds can be derived that have an increased specificity towards specific microorganisms. 362E (**Figure 1**) is a compound in pre-clinical

development as a novel therapeutic against *C. difficile*, as it is shows preferential activity against *C. difficile* PolC over those of other organisms *in vitro* [19,20].

PolC-inhibitors can cause a stress response and cell death after prolonged exposure. In *Bacillus subtilis*, this stress is characterized by a combination of DNA damage (SOS) response, and an SOS-independent pathway dependent on the DNA replication initiator DnaA [21,22]. In *Streptococcus pneumoniae* cells, devoid of an SOS-response, competence for genetic transformation is induced upon replication stress [23]. The response of *C. difficile* to this particular class of compounds is unknown.

In this study, we characterized aspects of the action of PolC-inhibitors towards *C. difficile*. Minimal inhibitory concentrations for HPUra and 362E were determined using agar dilution for a large collection of clinical isolates. Next, we investigated the effects of sub-inhibitory levels of PolC-inhibitors on growth of *C. difficile* in liquid medium and performed RNA sequencing (RNA-Seq) analyses to determine the transcriptional response to PolC-inhibitors in our laboratory strain 630Δ*erm*. Finally, marker frequency analysis was used to provide a mechanistic explanation for the observed up-regulated of origin-proximal genes under conditions of replication inhibition.

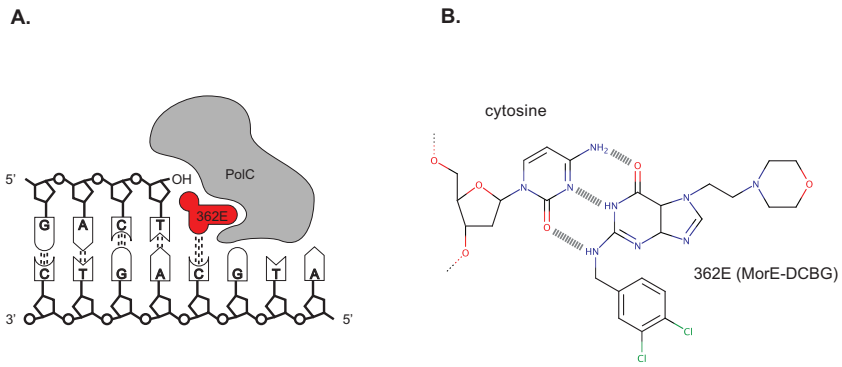


**Figure 1. Mechanism of action of the PolC-inhibitor 362E.**

**A.** Ternary complex of inhibitor 362E, DNA, and PolC.
**B.** H-bonding between inhibitor molecule 362E and a cytosine residue of DNA.

# Results

## 362E is a potent inhibitor of diverse clinical isolates of *C. difficile*

To date, reports on activity of PolC-inhibitors towards *C. difficile* are limited. For only 4 [19] and 23 [20] *C. difficile* strains minimal inhibitory concentrations were published, and no analysis was performed on possible differences in efficacy between various phylogenetic groups [8,24]. Therefore, we assessed the sensitivities of a diverse collection of *C. difficile* clinical isolates towards PolC-inhibitors and determined if 362E was indeed superior to the general PolC-inhibitor HPUra.

HPUra and 362E were tested by the agar dilution method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines for testing of antimicrobial susceptibility of anaerobes [25,26], against 363 *C. difficile* clinical isolates collected earlier in the framework of a pan-European study [6,27].

We found that 362E ($MIC_{50}$: 2 µg/ml; $MIC_{90}$: 4 µg/ml) demonstrates lower inhibitory concentrations than the general Gram-positive PolC-inhibitor HPUra ($MIC_{50}$: 16 µg/ml; $MIC_{90}$: 32 µg/ml) (**Figure 2**), consistent with previous *in vitro* activities observed against purified PolC [19].

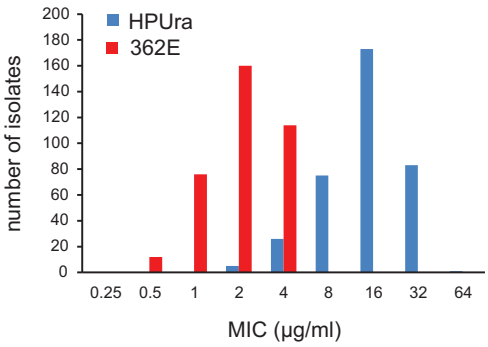


**Figure 2. Minimal inhibitory concentrations of PolC-inhibitors.**

MIC was determined by agar dilution according to CLSI standards [25] and is expressed in µg/mL. The distribution in the MIC for the collection of clinical isolates (*n*=363) is given for the PolC-inhibitors HPUra (blue) and 362E (red).

We observed no significant difference in 362E susceptibilities between clades (**Table 1**) and the different PCR ribotypes demonstrated a similar distribution in MIC values (*data not shown*).

**Table 1. Minimal inhibitory concentrations expressed in µg/ml of PolC-inhibitors stratified by clade**

| | $MIC_{50}$ | $MIC_{90}$ | Range | No. of isolates | PCR Ribotypes |
|---|---|---|---|---|---|
| **Clade 1** | | | | | |
| HPUra | 16 | 32 | 2-64 | 230 | 001, 002, 003, 005, 009, 010, 011, 012, 014, 015, 018, 025, 026, 029, 031, 037, 050, 051, 053, 056, 057, 064, 070, 081, 084, 087, 106 and 118 |
| 362E | 2 | 4 | 0,25-4 | | |
| **Clade 2** | | | | | |
| HPUra | 16 | 32 | 2-32 | 24 | 016, 019, 027, 075 and 208. |
| 362E | 2 | 4 | 0,5-4 | | |
| **Clade 3** | | | | | |
| HPUra | 16 | 32 | 4-32 | 7 | 023 |
| 362E | 4 | 4 | 1-4 | | |
| **Clade 4** | | | | | |
| HPUra | 8 | 16 | 2-16 | 9 | 017 |
| 362E | 2 | 4 | 1-4 | | |
| **Clade 5** | | | | | |
| HPUra | 16 | 32 | 4-32 | 43 | 033, 045, 078 and 126 |
| 362E | 1 | 2 | 0,5-4 | | |
| **Clade 6** | | | | | |
| HPUra | 4 | 4 | 4 | 1 | 131 |
| 362E | 4 | 4 | 4 | | |
| **Clade unknown** | | | | | |
| HPUra | 16 | 32 | 4-32 | 49 | 013, 024, 039, 046, 063, 090, 093, 097, 099, 101, 107, 110, 137, 139, 150, 154, 159, 161, 176, 202, 205, 207, 228, 229, 230, 231, 232, and 234 |
| 362E | 2 | 4 | 0,5-4 | | |

No growth at the highest concentration of compounds tested for either one of the PolC-inhibitors was observed among the collection of clinical isolates ($n$=363) (**Supplementary table 1**).

Notably, we observed only a 2-fold difference in $MIC_{50}$ and $MIC_{90}$, indicating that the compounds have similar activities against nearly all strains. In contrast, the Gram-negative obligate anaerobe *Bacteroides fragilis*, was resistant to both polymerase inhibitors under the conditions tested (MIC >265 µg/ml), as expected for an organism lacking PolC. The Gram-positive bacterium *Staphylococcus aureus*, which was included as a control for the activity of HPUra against this group of bacteria [16,28,29], was sensitive to both HPUra and 362E, with MIC values of 2 µg/mL and 1 µg/mL, respectively.

We conclude that 362E is highly active against diverse clinical isolates of *C. difficile*, and resistance is not a concern in currently circulating strains.

## Treatment with 362E leads to a pleiotropic transcriptional response

In order to determine the transcriptional response of PolC-inhibitors, we established the optimal concentration of both inhibitors which affected growth (sub-MIC levels) of *C. difficile* in liquid medium. The laboratory strain *C. difficile* 630Δ*erm* (PCR ribotype 012, multi-locus sequence type [MLST] Clade 1) [30,31] was grown in liquid medium with various amounts of HPUra (10-40 µg/mL) or 362E (0.25-8 µg/mL). We note that concentrations up to the $MIC_{90}$ (as determined by agar dilution) did not lead to a complete growth arrest in liquid medium in the time course of the experiment (growth was abolished at >2-fold MIC in liquid culture; *data not shown*). A difference in the MIC values between agar dilution and (micro)broth has been observed before [32]. The growth kinetics of *C. difficile* under the influence of varied concentrations of HPUra was marginally affected when using concentrations from 10-40 µg/ml, at approximately 80 percent of the non-treated culture. Growth kinetics of cultures containing PolC-inhibitor 362E at 1 to 8 µg/ml were similar and resulted in 30 to 40 percent reduced growth compared to that of the non-treated culture (**Figure 3**). For subsequent experiments, we used concentrations of PolC-inhibitors that result in a maximum reduction in growth of 30 percent compared to that of a non-treated culture (HPUra, 35 µg/ml; 362E, 4 µg/ml).

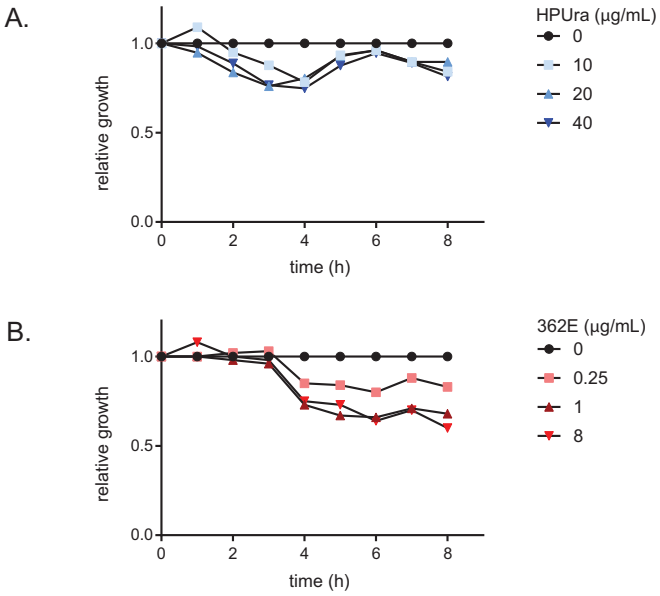


**Figure 3. Relative inhibition of growth by varying concentrations of PolC-inhibitors.**

**A.** Growth kinetics of a culture grown in the presence of the indicated amount of HPUra, relative to a non-treated culture.

**B**. Growth kinetics of a culture grown in the presence of the indicated amount of 362E, relative to a non-treated culture. Growth in liquid media was abolished at >2x the MIC (*data not shown*).

As described above, we established that growth of *C. difficile* is partially inhibited at certain concentrations of PolC-inhibitors. Slowing down or stalling of replication forks might lead to a stressed state, as observed for other organisms [21,23]. As nothing is known about the effect of replication inhibition on the physiology of *C. difficile*, we determined the transcriptional response to replication inhibition by sub-MIC levels of PolC inhibitors through strand-specific RNA sequencing (RNA-Seq).

*C. difficile* 630Δ*erm* was grown for 5h in medium with HPUra (35 µg/mL) or 362E (4 µg/mL) starting from an optical density at 600 nm ($OD_{600}$) of 0.05 after which cells were harvested for RNA isolation. Purified RNA was converted to cDNA and used for RNA-Seq as described in the Materials and Methods. For 362E, 722 genes were differentially expressed, of which 438 genes were up-regulated and 284

genes were down-regulated. The number of differentially expressed genes in HPUra-treated samples was ~2-fold lower, at 360, of which 124 genes were up-regulated and 236 genes were down-regulated. The full list of differentially regulated genes is available as **Supplementary Table 2** and the top 10 of up- and down-regulated genes are shown in **Table 2** (for HPUra) and **Table 3** (for 362E).

Here, we highlight three aspects of the results. First, we performed a Gene Set Enrichment Analysis (GSEA) [33] via the Genome2D web server [34] using the locus tags of the differentially regulated genes (**Supplementary Table 2**) as input. Among the genes up-regulated by 362E, there is a strong overrepresentation of those involved in translation, ribosome structure and ribosome biogenesis. Not unexpectedly, replication, recombination and repair processes are also affected. This suggests that genes from these pathways show a coordinated response in the presence of 362E. Among the genes down-regulated in the presence of 362E, the levels of significance for specific processes are generally much lower, suggesting that there is a more heterogeneous response among genes from the same pathway. Nevertheless, metabolic pathways (especially carbon metabolism and coenzyme A transfer) and tellurite resistance were found to be significantly affected. Strikingly, a GSEA analysis on lists of genes that are differentially expressed in the presence of HPUra revealed similar processes to be affected.

The findings from the GSEA analysis prompted us to evaluate the overlap in the lists of differentially regulated genes between the 362E and HPUra datasets in more detail. If the two compounds act via a similar mechanism, we expect a conserved response. Indeed, we observe that >90% of the genes that are up-regulated in the presence of HPUra compared to the non-treated condition, are also identified as up-regulated in the presence of 362E (**Figure 4A**). Though the overlap is not as strong for the down-regulated genes, we find that >30% of the genes affected by HPUra are also identified as affected by 362E (**Figure 4B**). Notably, the directionality of the response is conserved, as no genes were found to be up-regulated under one condition but down-regulated under the other condition. Based on these observations, we believe that the differentially expressed genes identified in this study are representative for a typical response to inhibition of PolC in *C. difficile*.

**Table 2. Top 10 up-and down regulated genes in HPUra treated samples**

**Top 10 significantly changed genes HPUra UP**

| RefSeq locus tag [1] | logFC | log CPM | LR | pvalue | adj_ pvalue | Fold | min FDR | Old locus tag [2] | Gene name [3] | Product | Protein ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CD630DERM_RS12480 | 11.16 | 3.84 | 9.8 | 1.8e-03 | 2.4e-02 | 2291.8 | 5.37 | CD630DERM_23052 | CD2305B | Hypothetical protein | WP_004454601.1 |
| CD630DERM_RS15730 | 6.02 | 12.24 | 39.2 | 3.9e-10 | 1.9e-07 | 64.9 | 22.30 | – | – | Hypothetical protein | WP_032506906.1 |
| CD630DERM_RS17185 | 5.12 | 8.69 | 48.8 | 2.9e-12 | 3.8e-09 | 34.8 | 27.97 | CD630DERM_32060 | CD3206 | Hypothetical protein | WP_003434302.1 |
| CD630DERM_RS17015 | 4.64 | 8.61 | 45.7 | 1.3e-11 | 1.0e-08 | 25.0 | 26.55 | CD630DERM_31740 | CD3174 | Type I glyceraldehyde-3-phosphate dehydrogenase | WP_003421962.1 |
| CD630DERM_RS17020 | 4.62 | 8.68 | 54.7 | 1.4e-13 | 2.7e-10 | 24.6 | 31.77 | CD630DERM_31750 | CD3175 | Transcriptional regulator | WP_003429564.1 |
| CD630DERM_RS00700 | 4.37 | 10.08 | 33.9 | 5.8e-09 | 2.1e-06 | 20.7 | 18.85 | CD630DERM_00801 | CD0080A | 50S ribosomal protein L29 | WP_003421158.1 |
| CD630DERM_RS00655 | 4.26 | 8.55 | 22.7 | 1.9e-06 | 1.4e-04 | 19.1 | 12.77 | CD630DERM_00720 | CD0072 | 30S ribosomal protein S10 | WP_011860633.1 |
| CD630DERM_RS16270 | 3.96 | 8.16 | 34.9 | 3.5e-09 | 1.4e-06 | 15.6 | 19.44 | CD630DERM_30310 | CD3031 | Transcriptional antiterminator | WP_003432034.1 |
| CD630DERM_RS19860 | 3.93 | 10.72 | 31.8 | 1.7e-08 | 4.1e-06 | 15.2 | 17.89 | CD630DERM_36630 | CD3663 | 30S ribosomal protein S6 | WP_003420522.1 |
| CD630DERM_RS00775 | 3.83 | 6.09 | 15.1 | 1.0e-04 | 2.7e-03 | 14.2 | 8.53 | CD630DERM_00930 | CD0093 | 50S ribosomal protein L14 | WP_009895197.1 |

**Top 10 significantly changed genes HPUra DOWN**

| RefSeq locus tag [1] | logFC | log CPM | LR | pvalue | adj_ pvalue | Fold | min FDR | Old locus tag [2] | Gene name [3] | Product | Protein ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CD630DERM_RS09945 | -14.85 | 4.52 | 17.0 | 3.7e-05 | 1.2e-03 | -29514.0 | 9.72 | CD630DERM_17940 | CD1794 | Hypothetical protein | WP_003430297.1 |
| CD630DERM_RS12730 | -14.52 | 4.33 | 17.3 | 3.1e-05 | 1.1e-03 | -23428.2 | 9.87 | CD630DERM_23550 | CD2355 | Thiol reductase thioredoxin | WP_003416870.1 |
| CD630DERM_RS00870 | -14.39 | 4.91 | 21.8 | 3.0e-06 | 1.9e-04 | -21483.7 | 12.35 | – | – | tRNA-Asn | – |
| CD630DERM_RS00255 | -14.35 | 5.48 | 45.8 | 1.3e-11 | 1.0e-08 | -20874.2 | 26.55 | – | – | tRNA-His | – |
| CD630DERM_RS00925 | -14.18 | 4.91 | 23.1 | 1.5e-06 | 1.3e-04 | -18514.8 | 12.89 | – | – | tRNA-Ser | – |
| CD630DERM_RS17905 | -14.02 | 5.11 | 26.3 | 3.0e-07 | 4.1e-05 | -16646.0 | 14.57 | – | | Hypothetical protein | WP_042741280.1 |
| CD630DERM_RS05300 | -14.01 | 4.27 | 12.7 | 3.7e-04 | 7.3e-03 | -16475.8 | 7.11 | CD630DERM_09230 | CD0923 | Hypothetical protein, putative phage protein | WP_021362052.1 |
| CD630DERM_RS00170 | -13.98 | 4.82 | 13.8 | 2.0e-04 | 4.5e-03 | -16156.5 | 7.78 | – | – | tRNA-Asn | – |
| CD630DERM_RS03115 | -13.96 | 5.24 | 56.8 | 4.8e-14 | 1.9e-10 | -15908.4 | 32.28 | CD630DERM_04981 | CD0498A | Hypothetical protein | WP_011860839.1 |
| CD630DERM_RS05160 | -13.92 | 3.89 | 15.3 | 9.1e-05 | 2.5e-03 | -15523.8 | 8.65 | CD630DERM_08960 | CD0896 | Hypothetical protein | WP_003418667.1 |

1 At time of analysis https://www.ncbi.nlm.nih.gov/nuccore/NZ_LN614756.1
2 https://www.ncbi.nlm.nih.gov/nuccore/LN614756,1
3 https://www.ncbi.nlm.nih.gov/nuccore/AM180355.1

**Table 3. Top 10 up- and down regulated genes in 362E treated samples**

**362E** **top 10 significant changed genes UP**

| RefSeq locus tag [1] | logFC | log CPM | LR | pvalue | adj_ pvalue | Fold | min FDR | Old locus tag [2] | Gene name [3] | Product | Protein ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CD630DERM_RS12480 | 11.31 | 3.84 | 9.9 | 1.6e-03 | 1.3e-02 | 2544.2 | 6.25 | CD630DERM_23052 | CD2305B | Hypothetical protein | WP_004454601.1 |
| CD630DERM_RS17185 | 7.73 | 8.69 | 87.6 | 8.1e-21 | 3.3e-17 | 211.9 | 54.77 | CD630DERM_32060 | CD3206 | Hypothetical protein | WP_003434302.1 |
| CD630DERM_RS17955 | 6.88 | 7.78 | 28.5 | 9.5e-08 | 5.0e-06 | 117.8 | 17.62 | CD630DERM_33520 | CD3352 | AraC family transcriptional regulator | WP_009891821.1 |
| CD630DERM_RS17950 | 6.87 | 9.82 | 31.9 | 1.6e-08 | 1.0e-06 | 117.3 | 19.88 | CD630DERM_33510 | CD3351 | ATP-dependent Clp protease proteolytic subunit | WP_003436212.1 |
| CD630DERM_RS15730 | 6.67 | 12.24 | 45.3 | 1.7e-11 | 4.0e-09 | 101.9 | 27.89 | - | - | Hypothetical protein | WP_032506906.1 |
| CD630DERM_RS00990 | 5.92 | 8.75 | 50.1 | 1.4e-12 | 5.7e-10 | 60.4 | 30.70 | CD630DERM_01090 | CD0109 | Anaerobic ribonucleoside-triphosphate reductase activating protein | WP_003432598.1 |
| CD630DERM_RS00655 | 5.65 | 8.55 | 35.0 | 3.2e-09 | 2.7e-07 | 50.3 | 21.80 | CD630DERM_00720 | CD0072 | 30S ribosomal protein S10 | WP_011860633.1 |
| CD630DERM_RS00985 | 5.50 | 8.94 | 66.3 | 3.8e-16 | 7.6e-13 | 45.2 | 40.27 | CD630DERM_01080 | CD0108 | Anaerobic ribonucleoside triphosphate reductase | WP_003436320.1 |
| CD630DERM_RS05405 | 5.49 | 10.12 | 43.5 | 4.2e-11 | 8.0e-09 | 45.0 | 26.89 | CD630DERM_09410 | CD0941 | Hypothetical protein | WP_009894743.1 |
| CD630DERM_RS15695 | 5.24 | 10.08 | 41.5 | 1.2e-10 | 2.0e-08 | 37.9 | 25.61 | CD630DERM_29250 | CD2925 | Hypothetical protein | WP_009894743.1 |

**362E** **top 10 significant changed genes DOWN**

| RefSeq locus tag [1] | logFC | log CPM | LR | pvalue | adj_ pvalue | Fold | min FDR | Old locus tag [2] | Gene name [3] | Product | Protein ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CD630DERM_RS05350 | -14.17 | 5.04 | 40.4 | 2.1e-10 | 3.1e-08 | -18382.7 | 24.94 | CD630DERM_09310 | CD0931 | Hypothetical protein | WP_011861014.1 |
| CD630DERM_RS05160 | -13.92 | 3.89 | 15.0 | 1.1e-04 | 1.6e-03 | -15523.8 | 9.33 | CD630DERM_08960 | CD0896 | Hypothetical protein | WP_003418667.1 |
| CD630DERM_RS05375 | -13.72 | 4.09 | 14.3 | 1.5e-04 | 2.0e-03 | -13454.4 | 8.95 | CD630DERM_09360 | CD0936 | Endonuclease | WP_011861020.1 |
| CD630DERM_RS15535 | -13.57 | 4.30 | 23.5 | 1.3e-06 | 4.4e-05 | -12181.6 | 14.47 | CD630DERM_28950 | CD2895 | Membrane protein | WP_011861046.1 |
| CD630DERM_RS13040 | -13.55 | 3.91 | 11.4 | 7.5e-04 | 7.1e-03 | -11987.2 | 7.15 | CD630DERM_24140 | CD2414 | PTS sorbose transporter subunit IIB | WP_003430855.1 |
| CD630DERM_RS05345 | -13.55 | 4.41 | 16.5 | 4.9e-05 | 8.2e-04 | -11969.7 | 10.25 | CD630DERM_09301 | CD0930A | Hypothetical protein | WP_011861013.1 |
| CD630DERM_RS11925 | -13.53 | 4.10 | 14.0 | 1.9e-04 | 2.3e-03 | -11799.9 | 8.76 | CD630DERM_21991 | CD2199A | 4Fe-4S ferredoxin | WP_003423406.1 |
| CD630DERM_RS01060 | -13.52 | 4.51 | 14.9 | 1.1e-04 | 1.6e-03 | -11718.7 | 9.33 | - | - | tRNA-Glu | - |
| CD630DERM_RS15350 | -13.44 | 3.89 | 15.3 | 9.1e-05 | 1.4e-03 | -11076.8 | 9.53 | - | - | Membrane protein | WP_003426729.1 |
| CD630DERM_RS06360 | -13.43 | 4.12 | 23.9 | 1.0e-06 | 3.7e-05 | -11012.2 | 14.72 | CD630DERM_11160 | CD1116 | Transposase | WP_011861118.1 |

1 At time of analysis https://www.ncbi.nlm.nih.gov/nuccore/NZ_LN614756.1
2 https://www.ncbi.nlm.nih.gov/nuccore/LN614756.1
3 https://www.ncbi.nlm.nih.gov/nuccore/AM180355.1

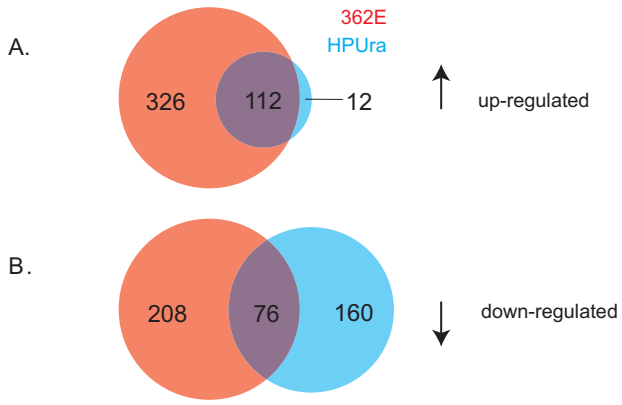


**Figure 4. Overlap in the transcriptional response to different PolC-inhibitors.**

**A.** Venn diagram of the number of genes up-regulated in the presence of 362E (red), in the presence of HPUra (blue) or under both conditions (overlapping region).

**B.** Venn diagram of the number of genes down-regulated in the presence of 362E (red), in the presence of HPUra (blue) or under both conditions (overlapping region). The size of the circles is proportional to the number of genes that showed differential expression.

Finally, we related the changes in transcription to genome location. *C. difficile* has a single circular chromosome and one origin of replication (*oriC*) from which the process of DNA replication occurs bi-directionally towards the terminus (*terC*) (**Figure 5A**). Though neither *oriC* nor *terC* has been definitively defined for *C. difficile*, it is assumed that *oriC* is located at or near *dnaA* (CD0001; CD630DERM_RS00005). The terminal region is generally located at the inflection point of a GC skew ([G - C]/[G + C]) plot. Such a plot places the *terC* region around 2.2Mb from CD0001, near the CD1933 (CD630DERM_RS10465) open reading frame (**Figure 5A**) [35].

We noted that the differential expression appeared to correlate with genome location (**Table 2, Table 3** and **Supplemental Table 2**), as many of the up-regulated genes have either low or high gene identifiers (CD numbers) indicative of an origin proximal location, conversely, many of the down-regulated genes appear to be located away from *oriC*. Though this correlation is not absolute, we observed a clear trend when plotting the mean fold change against genome location for all genes (**Figure 5B**).

Overall, our data show that inhibition of DNA replication by PolC-inhibitors causes a consistent and pleiotropic transcriptional response that is at least in part directly dictated by genome location.

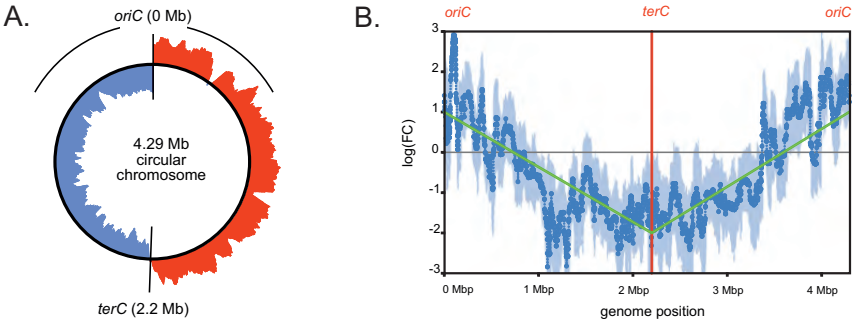


**Figure 5. Genome location correlates with differential expression upon PolC inhibition.**

**A.** Schematic representation of the chromosome of *C. difficile*. Higher than average GC skew ([G – C]/[G + C]) (red) and lower than average GC skew (blue) were calculated with DNAplotter [36]. Vertical lines indicate the position of the predicted origin (*oriC*) and terminus (*terC*) of replication. Arrows indicate the direction of replication.

**B.** Sliding window analysis (bins of 51 loci, step size 1) of the median log fold change (FC) projected on a linear genome map. The *oriC* of the circular chromosome is located on either size of the linear graph (0/4.29Mb), whereas *terC* is indicated with a vertical red line. The trend in log(FC) is highlighted using a green line. Light blue shading indicates the median absolute deviation of the mean [23].

6

## Gene dosage shift occurs at sub-inhibitory concentration of 362E PolC-inhibitor

A possible explanation for the relative up-regulation of *oriC*-proximal genes and down-regulation of *terC*-proximal genes is a gene dosage shift, due to the fact that PolC inhibition slows down replication elongation but does not prevent re-initiation of DNA replication [23]. To determine if this in fact occurs in *C. difficile* when replication elongation is inhibited, we performed a marker frequency analysis (MFA) to determine the relative abundance of an origin proximal gene relative to terminus proximal gene on chromosomal DNA isolated from treated and non-treated cells.

We designed quantitative PCR (qPCR) probes against the CD0001 and CD1931 regions, representing *oriC* and *terC*, respectively [31,35]. Using these probes, we could show that *C. difficile* demonstrates multi-fork replication in exponential growth phase and that the MFA assay detects the expected decrease in *oriC*:*terC* ratio when cells enter stationary growth phase (*data not shown*). Next, we analysed the effects of PolC-inhibitors on the *oriC*:*terC* ratio. When cells were treated with HPUra (35 µg/mL), the MFA showed a modest increase in *oriC*:*terC* ratio of 2.6-fold compared to non-treated cells. However, when cells were treated with 362E (4 µg/ml), the MFA showed a >8-fold increase in the *oriC*:*terC* ratio compared to non-treated cells (**Figure 6**). By contrast, such an increase was not observed for cells treated with metronidazole (0.25 µg/mL; a DNA damaging agent), fidaxomicin (0.00125 µg/mL; an RNA polymerase inhibitor) or surotomycin (0.625 µg/mL; a cell-wall synthesis inhibitor) (**Figure 6**) or chloramphenicol (2 µg/mL; a protein synthesis inhibitor) (*data not shown*). We conclude that inhibition of PolC-activity, but not the actions of any of the other tested antimicrobials, lead to a gene dosage shift in *C. difficile*.

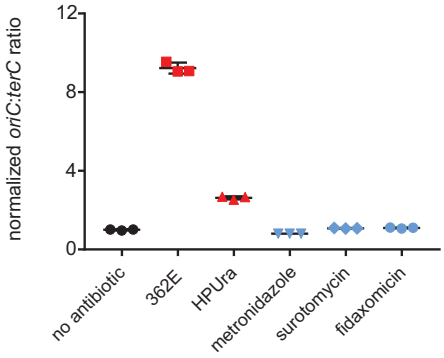


**Figure 6. Polymerase-inhibitors lead to an increase in *oriC*:*terC* ratio.**

A marker frequency analysis of the effects of sub-inhibitory amounts of polymerase inhibitors (red; HPUra: 35 µg/mL; 362E: 4 µg/mL) and three antibiotics with different modes of action (blue; metronidazole: 0.25 µg/mL; fidaxomicin: 0.00125 µg/mL; surotomycin: 0.625 µg/mL) compared to non-treated cells (black). Data points are averages of technical replicates ($n$=3). Black lines behind the data points indicate the average of the biological replicates ($n$=3) and whiskers indicate the standard deviation of the mean. Data have been normalized compared to the non-treated control. The mean of HPUra and 362E treated samples is statistically different from the other samples ($p$ <0.0001).

# Discussion

## Activity and specificity of 362E

Limited treatment options and reports of reduced susceptibility to current treatment [11,12,37] emphasise the necessity for the development of novel antimicrobials. More broadly, reducing the widespread and prolonged use of vancomycin, first-line therapy of severe CDI [9,10], may also contribute in minimizing the risk of inducing and shedding of vancomycin-resistant enterococci and staphylococci [19,20]. As CDI can be induced by use of broad-spectrum antibiotics [7], new antimicrobials ideally should only target *C. difficile*, thereby maintaining integrity of the colonic microbiota. In this study, we have tested the inhibitors HPUra and 362E which specifically target the PolC enzyme, which is essential for DNA replication. The majority of PolC-inhibitors target Gram-positive bacteria with low G+C content, but 362E has been reported to demonstrate increased specificity towards *C. difficile* PolC *in vitro* and showed promising results for the efficacy *in vivo*, based on a limited set of *C. difficile* strains [19,20]. The compound will progress to clinical trials in the near future (Acurx Pharmaceuticals, *personal communication*). The present study is the largest survey of the efficacy of HPUra and 362E against a large collection of clinical isolates consisting of many relevant PCR ribotypes to date. We have established that 362E demonstrated lower inhibitory concentrations than the general Gram-positive PolC-inhibitor HPUra in agar dilution experiments. The $MIC_{50}$ and $MIC_{90}$ of 362E are similar to those of antimicrobials commonly used to treat *C. difficile* infection (for metronidazole: $MIC_{50}$ = 2 µg/mL and $MIC_{90}$ = 4 µg/mL; for vancomycin $MIC_{50}$ = 2 µg/mL and $MIC_{90}$ = 4 µg/mL [20]). We did not detect a significant difference in MICs between clades and ribotypes, demonstrating that PolC-inhibitors have the potential to be used as treatment for the majority of – if not all – circulating *C. difficile* strains. This includes the epidemic types of PCR ribotype 027 and 078 [8,38]. These results are in line with other work that demonstrated only 2- to 4-fold differences in antimicrobial susceptibility between different clades for metronidazole, fidaxomicin, and semi-synthetic thiopeptide LFF571 [27]. In the course of our experiments, we did not find any strains that grew at the highest concentrations of either HPUra or 362E tested, suggesting that resistance against these particular PolC-inhibitors is

rare or absent in *C. difficile*. PolC-inhibitors are competitive inhibitors of polymerase activity by binding in the active site. Mutations that abolish binding of HPUra or 362E are likely to affect the essential enzymatic activity of the polymerase and for that reason unlikely to occur *in vivo*. A single mutation (*azp-12*) has been described in *B. subtilis* that confers resistance to HPUra [39]. This T>G transversion results in the replacement of a serine with an alanine in the highly conserved PFAM07733 domain of the polymerase [40]. To our knowledge, it is unknown whether this mutation prevents binding of HPUra to PolC of *B. subtilis*. Few other mutations have been described that confer resistance against other PolC-inhibitors [41,42]. It will be of interest to see if similar mutations in *C. difficile* result in resistance to HPUra and/or 362E and what the effect is on binding of these compounds to *C. difficile* PolC.

In our experiments, we included *S. aureus* as a control for the activity of HPUra against Gram-positive bacteria. Surprisingly, we found that *S. aureus* was highly sensitive to both HPUra and 362E, and even more so than *C. difficile*. The reasons for this sensitivity are unknown. It is conceivable that 362E also targets PolC of *S. aureus* and if so, this information could be used to further characterize the interaction of 362E with PolC. Alternatively, 362E may also affect the activity of the other PolIII-type polymerase, DnaE. PolIII-inhibitors can affect PolC, DnaE or both [41], though *in vivo* activity appears to correlate with PolC-inhibition. Both *C. difficile* and *S. aureus* possess PolC and DnaE polymerase, but the DnaE enzymes belong to different families (DnaE1 in *C. difficile* and DnaE3 in *S. aureus*). The phylogenetic split coincides with the taxonomic division as DnaE3- polymerases are found in the class Bacilli (which includes *S. aureus*), whereas the DnaE1 polymerase are present in the classes Clostridia and Negativicutes [43]. To test whether a difference in activity of 362E towards different DnaE-type polymerases explain the increased sensitivity of *S. aureus* compared to *C. difficile*, the activity of 362E towards purified DnaE from both organisms should be determined. Finally, 362E may have off-target effects in *S. aureus* unrelated to its replication-inhibitory activity.

Though it is clear that 362E inhibits *C. difficile* efficiently and shows limited activity towards certain other anaerobes [19], these findings highlight that it is necessary to perform additional (microbiome) studies to more clearly define the antimicrobial spectrum of this compound. It also shows that 362E may have therapeutic potential outside treatment of CDI.

## Regulators of the transcriptional response to *PolC inhibitors*

The present study is the first to describe the transcriptional response of *C. difficile* to inhibition of DNA replication. We find that ~200 genes show differential expression under conditions of PolC-inhibition by both HPUra and 362E, compared to non-treated cells (**Supplemental Table 2, Figure 4**). When considering only 362E, approximately 13 percent of all genes in the genome show statistically significant altered transcription. We demonstrate that this large reprogramming of transcription is likely to be caused directly by a gene dosage shift (**Figure 5** and **6**).

The main limitation of our study is the fact that we cannot conclusively identify one or more mechanisms that explains the transcriptional response. There are several reasons for this.

First, our list of differentially regulated genes includes several putative regulators; sigma factors (including *sigE*, *sigG* and *sigH*), transcription factors and anti-terminators. The relatively long induction time (5h) at sub-MIC levels of antimicrobials may have contributed to secondary effects, through one or more of these regulators. Though shorter induction times are thought to provoke more compound-specific responses [44], we did observe a highly consistent transcriptional signature with both HPUra and 362E (**Supplemental Table 2, Figure 4**).

Second, major stress response pathways are poorly characterized in *C. difficile*. On the basis of experiments in other organisms [21,23,45-47], we expect that inhibition of DNA replication inhibition might possibly induce an SOS response (LexA) [48], a DnaA-dependent transcriptional response [21], and possibly a heat shock response (HrcA/CtsR) [50] and/or a general stress response (SigB) [49]. Putative LexA-regulated genes of *C. difficile* were identified *in silico* [51] and some of these (such as the *uvr* excinuclease and 30S ribosomal protein S3) were differentially expressed in our dataset (**Supplemental Table 2**). However, pleiotropic phenotypes have been described for a *C. difficile lexA* mutant [52] and it is likely that other LexA targets exist. To date, no genes have been identified that are regulated by DnaA in *C. difficile* and direct regulation of genes through the other pathways has not been demonstrated. No mutants of *hrcA* or *ctsR* have been described for *C. difficile*, but transcriptome and proteome analyses have been performed with heat shocked cells 42°C [53], or 41°C [54-56]. Similarities between these datasets and our data include genes encoding

proline racemase (*prdF*), chaperones (*groEL*, *groES*), thioredoxin systems (*trxA*, *trxB*) and Clp-proteases (*clpC*, *clpP*). In contrast to most anaerobic Gram-positive organisms, *C. difficile* encodes a homologue of the general stress response sigma factor, $\sigma^B$ [49,57]. A transcriptome analysis comparing a *sigB* mutant versus wild type cells was recently published [58]. Strikingly, we find ~35% of the genes (20/58) identified as involved in stress response under the control of $\sigma^B$ to be differentially expressed in our 362E dataset (including many non-characterized genes), suggesting that the response to DNA replication inhibition is at least partially dependent on $\sigma^B$. It should be noted that the *sigB*-operon was not differentially expressed. This phenomenon has been observed before in other organisms and is attributed to a persistent upregulation of *sigB* followed by a persistent response of $\sigma^B$-dependent gene expression [50,53,55]. In our experiment, cells were grown for 5 hours at sub-MIC levels of inhibitors, making it unlikely that altered *sigB* transcription would still be detected. Alternatively, $\sigma^B$ activity might be regulated post-translationally.

Many parameters (such as the medium used, cell density, concentration of antibiotics, and protocol used to arrest transcription between cell harvest and lysis) can influence overall transcription signatures and can also govern an incomplete overlap between our data and the stress regulons determined by others [44]. To conclusively demonstrate an involvement for any of these pathways in the response of *C. difficile* to replication inhibition, independent validation of the results from the RNA-Seq analysis is necessary. Validation of RNA-Seq data frequently using qPCR on the same RNA will not validate any biological conclusions but validates the technology [59]. For that reason, our current efforts are directed to the construction of luciferase reporter-fusions [60] that can be used to study promoter activity under different conditions and in different mutant backgrounds.

## Genome location contributes to the transcriptional response to PolC-inhibition

Our analysis of differential regulation in relation to genome location revealed a striking pattern of relative up-regulation for *oriC*-proximal genes, and down-regulation for *terC*-proximal genes under conditions of PolC inhibition (**Figure 5**). Antimicrobials directed at DNA replication in bacteria have a profound negative effect on the processivity of replication forks, though initiation of DNA replication is

not or only marginally affected [23, 39]. As a consequence, the presence of multiple replication forks simultaneously increases the copy numbers of genes located in close proximity of the origin of replication, and such a gene dosage differences can result in functionally relevant transcriptional differences, either directly or indirectly [15]. We found an increase of *oriC*:*terC* ratio when performing MFA on chromosomal DNA of cells subjected to a sub-inhibitory concentration of 362E (and HPUra, albeit less pronounced) (**Figure 6**), consistent with findings in other organisms [23].

An example of direct regulation by gene dosage can be found in *Vibrio*, for instance, where the location of ribosomal protein clusters close to the origin is crucial for fast growth, because increased copy number under condition of multi-fork replication allows for higher expression levels [61]. We note that ribosomal gene clusters are up-regulated when DNA replication is inhibited in our experiments (**Table 2, Table 3, Supplemental Table 2**), suggesting that a similar mechanism may be active in *C. difficile*.

An example of indirect regulation as a result of gene dosage is the induction of competence genes in *S. pneumoniae* [23]. Competence is believed to be a stress response in this organism, that lacks a canonical ($\sigma^B$-dependent) stress response pathway. Key regulatory genes for competence development are located close to the origin, and replication inhibition therefore leads to the induction of origin distal competence genes [23]. In our experiments, the large overlap with the proposed $\sigma^B$ regulon [58] and the origin proximal location of the *sigB* operon (8.5kb-10kb) suggest that part of the transcriptional response to PolC-inhibition can be explained by an indirect gene dosage effect. The positioning of stress-response regulators close to *oriC* may therefore be a conserved strategy in bacteria to respond to DNA replication insults, independent of the nature of the regulator.

Though it is likely that an increase in gene copy number leads to an increase in transcription of these genes, it is less clear whether this is the case for the observed down-regulation. Most methods of normalization for transcriptome analyses are based on the assumption that there is no overall change in transcription or that the number of transcripts per cells is the same for all conditions and this may not be the case when a global copy number shift occurs [15]. Absolute transcript levels for down-regulated genes might therefore be similar under both conditions (but lower, relative to *oriC*-proximal transcripts).

It is interesting that certain processes are highly enriched in the list of genes up-regulated under conditions of PolC-inhibition (most notably ribosome function and DNA-related functions), whereas this is less so for the down-regulated genes. This suggests that pathways susceptible to replication-dependent gene dosage effects demonstrate a functional clustering of genes near *oriC*, whereas clustering of genes belonging to specific pathways in the *terC*-proximal region is less pronounced. Indeed, most ribosomal gene clusters in *C. difficile* are located close to the origin of replication [31,57] and many genes involved in DNA replication and repair are located in these regions. Consistent with this, positioning of genes involved in transcription and translation close to the origin appears to be under strong selection as such genomes tend to be more stable [65].

In conclusion, both direct and indirect effects of gene dosage shifts are likely to contribute to the transcriptional response of *C. difficile* to replication inhibition.

## Materials and Methods

### Agar dilution

HPUra and 362E were tested against a collection of *C. difficile* clinical isolates. 375 clinical isolates have been collected during the ECDIS study [6]. All strains were characterized by PCR ribotyping [62] and by PCR to confirm the presence of genes encoding toxins A, B and binary toxin [63-65]. Of the 375 clinical isolates, we excluded stocks that were found to contain more than one strain and isolates that could not be re-cultured. Testing was therefore performed on 363 isolates (**Supplemental Table 1**). *C. difficile* ATCC 700057, *B. fragilis* ATCC 25285 and *S. aureus* ATCC 29213 were used as controls [66].

The strains were tested for the different concentrations of antimicrobial using the agar dilution method according to Clinical & Laboratory Standards Institute guidelines [25]. In short, the antimicrobials were diluted into Brucella Blood Agar (BBA) supplemented with hemin and vitamin K1. Bacterial isolates were cultured on blood agar plates and after 24 hours re-suspended to a turbidity of 0.5 McFarland in phosphate buffered saline (PBS). The strains were inoculated onto BBA solid media containing the PolC-inhibitors using multipoint inoculators to a final con-

centration of $10^4$ CFU per spot. Each series of antimicrobial agents was tested from the lowest concentration to the highest concentration. Two control plates without antibiotics were inoculated to control for aerobic contamination and purity of anaerobic growth. At the end of the final series, two control plates were inoculated to verify the final organism viability and purity. Plates were incubated anaerobically in a variable atmosphere cabinet (VA1000, Don Whitley Scientific) and the MICs were determined after 24 and 48 hours.

## Sub-MIC determination

*C. difficile* 630Δ*erm* [30,31] was grown in 20 mL Brain Heart Infusion (Oxoid) supplemented with 0.5% yeast extract (Sigma-Aldrich) (BHI/YE) starting from an optical density measured at 600 nm ($OD_{600}$) of 0.05 using an exponentially growing starter culture (3 biological replicates per concentration). To determine the effects on growth of sub-inhibitory concentration of 362E, cells were cultured in the presence of the following concentrations; 0.25, 0.5, 1, 2, 4, and 8 µg/mL 362E and compared to an untreated culture. To determine the effects on growth of sub-inhibitory concentration of HPUra, cells were cultured in the presence of the following concentrations; 10, 20, 40, 80 µg/mL HPUra and compared to an untreated culture. The $OD_{600}$ was monitored every hour until stationary phase was reached.

## Marker Frequency analysis

*C. difficile* 630Δ*erm* [30,31], was grown in 20 mL BHI/YE with sub-MIC amounts of antimicrobials (HPUra: 35 µg/mL; 362E: 4 µg/mL; metronidazole: 0.25 µg/mL; fidaxomicin: 0.00125 µg/mL, surotomycin: 0.625 µg/mL), starting from an $OD_{600}$ of 0.05 using an exponentially growing starter culture. We confirmed that these concentrations did not lead to a >30% reduction in growth compared to non-treated cultures (**Figure 3** and *data not shown*). In parallel, cultures were grown without inhibitors from the same starter culture. All conditions were performed in biological triplicates. After 5 hours, 1mL cells was harvested ($OD_{600}$ ~ 0.5), and stored at -20°C. Isolation of chromosomal DNA was performed the next day with the QIAamp DNA Blood Mini kit (Qiagen) according to the instructions of the manufacturer. Marker frequency analysis (MFA) was performed to assess the relative abundance of origin proximal

genes relative to terminus proximal genes. As a proxy for *oriC*, a probe was designed that targets the CD0001 region (CD0001-probe-FAM). By using plots of the GC skew ([G - C]/[G + C]) generated using DNAPlotter [36], the approximate location of the terminus region for the *C. difficile* chromosome was determined and a probe targeting this region (CD1931) was designed (CD-1931-probe-TXR). Probe design was performed with Beacon Designer™ (Premier Biosoft, Palo Alto CA, USA). Real-time PCR reactions were performed on a Biorad CFX96™ real-time PCR detection system (95°C 15 m, 39 × (94°C 30 s + 55°C 30 s + 72°C 30 s). Sequences for primers and probes are listed in **Table 4**.

**Table 4. Oligonucleotides and probes used in this study**

| Name | Sequence (5' – 3' ) | Description |
|---|---|---|
| CD-0001- F | GAGACAAGAATTGCTATACTTA | Forward primer CD0001 MFA (oriC) |
| CD-0001- R | CAACCACTCTAGTTAATGC | Reverse primer CD0001 MFA (oriC) |
| CD-0001-probe-FAM | CTCAACTAGAACGTATAGATGTGCCAA | Probe CD0001 MFA (oriC) |
| CD-1931- F | GCAGGAATTTTAGATGAAGA | Forward primer CD1931 MFA (terC) |
| CD-1931- R | GGCTGAAGTCTTATTAAATTTC | Reverse primer CD1931 MFA (terC) |
| CD-1931-probe-TXR | CCTCTTAACTGTAGCAGATTCACCAT | Probe CD1931 MFA (terC) |

For each biological replicate, three technical replicates were performed. Amplification efficiency was determined using standard curves obtained from DNA late stationary phase cells of strain 630Δ*erm*, for which an *oriC*:*terC* ratio of 1 was assumed. RT-PCR results from antibiotic treated cells were normalized to the *oriC*:*terC* ratio of DNA samples (3 biological replicates) from non-treated cells. Calculations were performed in Microsoft Office Excel 2010, plotted using Prism 7 (GraphPad) and prepared for publication in Corel Draw Suite X8. Significance was determined using a One-way ANOVA and a Tukey's test for multiple comparisons (GraphPad).

## Growth and RNA isolation for RNA-Seq

For RNA-Seq analysis, *C. difficile* 630Δ*erm* was grown for 5h in BHI medium with HPUra (35 µg/mL) or 362E (4 µg/mL) starting from an $OD_{600}$ of 0.05 using an

exponentially growing starter culture, after which cells (3mL) were harvested for RNA isolation. RNA isolation was performed with NucleoSpin© RNA kit (Macherey-Nagel). Although the kit includes on column rDNAse digestion, a second treatment was performed in solution and RNA was precipitated and recovered by NaAc precipitation to remove residual DNA. Concentration determination and quality control (16S/23S ratio and RNA integrity number [RIN]) was performed with a fragment analyser (Agilent bio-analyzer), according to the instructions of the manufacturer. Samples with a RIN>9 and 16S/23S ratio >1.4 were submitted for analysis by RNA-Seq.

## RNA-Seq

RNA-Seq was performed at a commercial provider (GenomeScan, Leiden, The Netherlands). In short, the NEBNext Ultra Directional RNA Library Prep Kit for Illumina was used to process the samples. Sample preparation was performed according to the protocol "NEBNext Ultra Directional RNA Library Prep Kit for Illumina" (NEB #E7420S/L). Briefly, after selective removal of rRNA (Ribo-Zero rRNA Removal Kit for Gram-Positive Bacteria) and fragmentation of the mRNA, cDNA synthesis was performed. cDNA was ligated to the sequencing adapters and the resulting product was PCR amplified. Clustering and DNA sequencing using the Illumina NextSeq 500 platform was performed according manufacturer's protocols. A concentration of 1.5 pM of DNA was used. Image analysis, base calling, and quality check was performed with the Illumina data analysis pipeline RTA vl.18.64 and Bcl2fastq v2.17. Per sample, four technical replicates were included in the RNA-Seq experiment. In case of insufficient reads, the sample was re-run on another flow cell to reach satisfactory quantities (≥ 20 M).

### Analysis of RNA-Seq data

Analysis of the data was performed using T-REx, a user-friendly webserver which has been optimized for the analysis of prokaryotic RNAseq-derived expression data [67]. The pipeline requires raw RNA expression level data as an input for RNA-Seq data analysis. For data normalisation and determination of the genes, the factorial design statistical method of the RNA-Seq analysis R-package EdgeR is implemented in the T-Rex pipeline. Some samples displayed incomplete rRNA depletion and rRNA mapping reads had to be removed manually prior to analysis.

To analyse the genome-wide pattern in differential gene expression a sliding window analysis was performed essentially as described[23]. In short, genome locations (start of the locus tag) were coupled to the locus tags in the T-Rex output. Next, the median log(FC) was calculated for bins of 51 loci with a step size of 1. For each bin of $[X_1, X_2...X_{51}]$ the median absolute deviation of the median (MAD=median($|X_i$-median(X)|) was calculated as a robust indication of the distribution around calculated median values. Calculations were performed and three curves (median, median-MAD and median+MAD) were plotted in Microsoft Office Excel 2010 and the graph was prepared for publication using Adobe Photoshop CC and Corel Draw Suite X8.

A GSEA analysis [33] was performed via the Genome2D webserver [34], using our reference genome sequence for *C. difficile* 630Δ*erm*, GenBank identifier LN614756.1 (listed in Genome2D as "Clostridioides_difficile_630Derm") [31]. As input a single list of locus tags was used of either up- or down regulated genes. The output was copied to Microsoft Excel 2010. The single list column was split, and a column was inserted to calculate the significance of the overrepresentation using the formula "(# hits in list/ClassSize)*-log(p-value; 2)" to allow for sorting of the output of the GSEA analysis by significance.

## Acknowledgements

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### Supplemental Information

Supplemental tables associated with this chapter can be found online at bioRxiv (pre-print server for Biology) via the following link: https://bit.ly/2mqcoLw

**Supplemental Table 1.** Characteristics of the clinical isolates used in the agar dilution experiments.

Supplemental Table 1_ agar dilution (as XLSX)

**Supplemental Table 2.** Lists of the genes that are differentially expressed in the presence of PolC inhibitors compared to non-treated cells.

Supplemental Table 2_ Significant (as XLSX)

**Supplemental Table 3A.** SigB-dependent stress response genes differentially regulated in the 362E dataset.

Supplemental Table 3_ SigB (as XLSX)

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